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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The BRCA1 protein participates in multiple cellular processes, and we resolved four BRCA1-containing complexes. One of these complexes observed after treating with hydroxyurea, the HUIC (hydroxyurea induced complex) is involved with DNA damage. Following HU treatment, BRCA1 content decreased in the holo-pol (RNA polymerase II holoenzyme) and in the large complex we refer to as the Fraction 5 complex, and BRCA1 was redistributed to the HUIC. By overexpressing epitope-tagged BRCA1, full-length and deletion mutants, we characterized BRCA1 association with these complexes. We found that BARD1 is an integral holo-pol component and that the BARD1 binding domain in the BRCA1 amino-terminus is important for BRCA1 association with the holo-pol. The carboxy-terminus of BRCA1 is important for association with holo-pol, but deletion of the BRCA1 carboxy terminus resulted in low Pol II content and the absence of Pol II from the holo-pol. The HUIC was shown not to contain a number of holo-pol components, but is associated with BARD1. In a separate chromatographic fraction, BRCA1 was found associated with the Rad50/Mre11/Nbs1 complex. These data suggest that BRCA1 participates in multiple cellular processes by multiple protein complexes and that the BRCA1 content of these complexes is altered following DNA damage.

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INTRODUCTION

Mutations in the *BRCA1* tumor suppressor gene are associated with about 50% of all familial breast cancers (Venkitaraman, 2000; Zheng et al, 2000). Emerging data indicate that BRCA1 is likely to serve as an important central component in multiple biological pathways that regulate transcription, repair of DNA damage, cell cycle, polyadenylation of mRNAs, and chromatin remodeling (Deng et al, 2000; Kleiman and Manley, 2001; Bochar et al, 2000; Parvin, 2001).

BRCA1 has been found associated with multiple polypeptides and several BRCA1-containing complexes have been purified using different methods, whose functions are likely to be different (Deng et al, 2000; Bochar et al, 2000; Cantor et al, 2001). BRCA1 protein dynamically changes its subcellular localization dependent on the cell cycle or whether the genome has been damaged (Scully et al, 1997a; Scully et al, 1997b). It is unknown whether these changes in subcellular position reflect changes in BRCA1 protein complexes.

Our laboratory already established biochemical purification strategy that can partially purify RNA polymerase II holoenzyme (holo-pol) (Scully et al 1997c; Neish et al 1998). Using this system, we have reported that BRCA1 is associated with holo-pol. This large protein complex regulates the synthesis of all mRNA in the cell, and the association of BRCA1 with this complex suggests a role in transcription. We have identified that by treating cells with hydroxyurea (HU), a new BRCA1-containg complex is induced, which we call the HUIC.

In this study, we are characterizing the HUIC and other BRCA1-containing complexes. Our experiments will make clear whether all of these multiple processes are due to one biochemical mechanism or due to multiple mechanisms with BRCA1 functioning in multiple protein complexes.

BODY

Expression of tagged BRCA1 using retrovirus:

We prepared retrovirus vectors subcloned HA-tagged BRCA1 genes and confirmed that they produce BRCA1 proteins. We infected HeLa cells with these constructs and select for retrovirus containing cells using puromycin. We cloned stable transformants that express HA-BRCA1 and grew these clones under puromycin selection. We analyzed about 170 independent clones and we found several with low expression of the HA-tag on a protein consistent with BRCA1. We prepared extracts from these cells but found that they all grew poorly. We were able to prepare whole cell extracts, which we fractionated

using the Biorex70 ion exchange matrix, and these fractions were subjected to sucrose gradient sedimentation according to our standard protocols. These protein samples were analyzed by Western blots for HA-epitope. The expression level of HA-BRCA1 in the cell was too low to analyze and purify BRCA1-containing complexes using immunopurification by HA-epitope. We note that BRCA1 is a tumor suppressor and would be expected to inhibit cell growth. Several groups have reported already that overexpression of BRCA1 cause cells growth suppression and cell cycle arrest. Combining these results, we have concluded that it was impossible to get high expressor clones of HA-BRCA1.

Expression of tagged BRCA1 using recombinant adenovirus:

We changed the expression system from stable expression to transient expression. We constructed adenovirus vectors to express HA-tagged BRCA1 of full-length and several deletion mutants.

We achieved high levels of HA-BRCA1 expression in large scale culture using the adenovirus system. Full-lengh BRCA1 tagged with the HA-epitope at the either the amino or carboxy terminus was expressed highly, and these were purified from extracts. The expressed tagged BRCA1 protein purified similarly as did the endgenous BRCA1 protein. In addition, four large deletion mutants were expressed, and three of these copurified with the holo-pol complex and the third did not. By these results we mapped key protein domains for BRCA1 interaction with the holo-pol.

Using the amino terminal deleted mutant (Δ1-302) of BRCA1 and the carboxy terminal deleted mutant (Δ1527-1863), we characterized the association of BRCA1 and the holo-pol. We found that BARD1 is an integral holo-pol component and that the BARD1 binding domain in the BRCA1 amino-terminus is important for BRCA1 association with the holo-pol. Consistent with earlier observations, the carboxy-terminus of BRCA1 is important for association with holo-pol, but, surprisingly, deletion of the BRCA1 carboxy terminus resulted in low Pol II content and the absence of Pol II from the holo-pol.

We have also identified a third complex in this step of the purification which we refer to as the Fraction 5 complex. Our biochemical purification system also separarates the BRCA1-Rad50- Mre11-NBS1 complex (Zhong et al,1999) from the other three complexes. We have thus resolved four different BRCA1-containing complexes, one of which is only observed following HU treatment of cells.

Characterization of the HUIC using adenovirus expressed HA-BRCA1:

The central aim of this project is to characterize the BRCA1-containing complex induced by hydroxyurea treatment of cells, the HUIC. We had used HU to block cells in the S-phase of the cell cycle and to identify new complexes with which BRCA1 associates. We found most of the endogenous BRCA1 is in the HUIC. To apply the recombinant adenovirus expressed HA-BRCA1 to the characterization of the HUIC, we expressed full-length HA-BRCA1 in cells, treated with hydroxyurea, and fractionated proteins. To our great surprise, we could not detect this BRCA1 complex. Since overexpression of BRCA1 is known to cause a G1 arrest, we infer that overexpressing full-length BRCA1 might be imcompatible with BRCA1 association with the S-phase specific HUIC complex. In contrast, one of our deletion mutants, HA-BRCA1(Δ775-1292), which did not suppress growth of cells, did reveal the presence of the S-phase specific HUIC on sucrose gradient sedimentation analysis.

Using this deletion mutant, we characterized this HUIC by immunoprecipitaion using antibody specific for HA-epitope, DNA repair related protein, Mre11, and holoenzyme specific component, SRB7. These analyses revealed that the HUIC is different from the holo-pol and different from the Rad50/Mre11/Nbs1 complex. We found that the HUIC contains BARD1(BRCA1-associated RING domain), a key BRCA1 interacting protein.

We are now purifying the HUIC by immunopurification. Using silver stained protein gels, we are optimizing conditions to scale up to analyze components of this complexes by mass spectroscopy.

KEY RESEARCH ACCOMPLISHMENTS

In this study we purified four distinct BRCA1-containing complexes; the BRCA1-Rad50 complex, the Fraction 5 complex, the holo-pol complex, and the HUIC. We demonstrated the HUIC, which is different from holo-pol, and we showed that the HUIC contains BARD1.

REPORTABLE OUTCOMES

We have submitted a manuscript describing the identification of the four different BRCA1-containing complexes. We anticipate in the next two years of this fellowship we will characterize the polypeptide compositions of these complexes and identify their biochemical activities.

CONCLUSIONS

In these experiments we resolved four distinct BRCA1-containing complexes. One of these complexes which is observed after treating cells with hydroxyurea, the

HUIC (hydroxyurea induced complex) is involved with the response to DNA damage. Following HU treatment of cells, BRCA1 content decreased in the holo-pol (RNA polymerase II holoenzyme) and in the large complex we refer to as the Fraction 5 complex, and BRCA1 was redistributed to the HUIC. By overexpressing epitope-tagged BRCA1, full-length and deletion mutants, we characterized BRCA1 association with the holo-pol and with these three other complexes. We found that BARD1 is an integral holo-pol component and that the BARD1 binding domain in the BRCA1 amino-terminus is important for BRCA1 association with the holo-pol. Consistent with earlier observations, the carboxy-terminus of BRCA1 is important for association with holo-pol, but, surprisingly, deletion of the BRCA1 carboxy terminus resulted in low Pol II content and the absence of Pol II from the holo-pol. Using tagged BRCA1 expressing cells, the HUIC was shown not to contain a number of holo-pol components, but is associated with BARD1. Lastly, in a separate chromatographic fraction, BRCA1 was found associated with a complex containing Rad50/Mre11/Nbs1. These data suggest that BRCA1 participates in multiple cellular processes by multiple protein complexes and that the BRCA1 content of these complexes is dynamically altered following DNA damage.

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Redistribution of BRCA1 among four different protein complexes following DNA damage

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The abbreviations used are: Pol II, RNA polymerase II; holo-pol, RNA polymerase II holoenzyme; SRB, suppressor of RNA polymerase B mutations; BARD1, BRCA1-associatead RING domain; HU, hydroxyurea; HUIC, hydroxyurea induced complex; Nbs, Nijmegen breakage syndrome; BASC, BRCA1-associated genome surveillance complex

ABSTRACT

The BRCA1 protein is known to participate in multiple cellular processes, and in these experiments we resolved four distinct BRCA1-containing complexes. One of these complexes has not been described previously, and we observe it after treating cells with hydroxyurea, suggesting that this new complex, the HUIC (hydroxyurea induced complex) is involved with the response to DNA damage. Following HU treatment of cells, BRCA1 content decreased in the holo-pol (RNA polymerase II holoenzyme) and in the large complex we refer to as the Fraction 5 complex, and BRCA1 was redistributed to the HUIC. By overexpressing epitope-tagged BRCA1, full-length and deletion mutants, we characterized BRCA1 association with the holo-pol and with these three other complexes. We found that BARD1 is an integral holo-pol component and that the BARD1 binding domain in the BRCA1 amino-terminus is important for BRCA1 association with the holo-pol. Consistent with earlier observations, the carboxy-terminus of BRCA1 is important for association with holo-pol, but, surprisingly, deletion of the BRCA1 carboxy terminus resulted in low Pol II

content and the absence of Pol II from the holo-pol. Using tagged BRCA1 expressing cells, the HUIC was shown not to contain a number of holo-pol components, but was associated with BARD1. Lastly, in a separate chromatographic fraction, BRCA1 was found associated with a complex containing Rad50-Mre11-Nbs1. These data suggest that BRCA1 participates in multiple cellular processes by multiple protein complexes and that the BRCA1 content of these complexes is dynamically altered following DNA damage.

INTRODUCTION

Mutations in the *BRCA1* tumor suppressor gene are associated with about 4% of all breast cancers and about 50% of all familial cases (1,2). Emerging data indicate that BRCA1 is likely to serve as an important central component in multiple biological pathways that regulate transcription, repair of DNA damage, cell cycle, polyadenylation of mRNAs, and chromatin remodeling (3-6). It is not clear whether all of these processes are due to one

biochemical mechanism or due to multiple mechanisms with BRCA1 functioning in multiple protein complexes.

BRCA1 has been found associated with multiple polypeptides, including BARD1, which binds to the amino terminus of BRCA1 (7). Both BRCA1 and BARD1 proteins contain a RING finger motif and BRCT repeat. The BRCA1-BARD1 complex interacts with a polyadenylation factor, CstF-50 (cleavage stimulation factor), suggesting a link between the regulation of polyadenylation of mRNA and DNA repair (4). BRCA1 has ubiquitin ligase activity and, in association with BARD1, ubiquitin ligase activity is high (8-10). Direct specific interaction between BRCA1 and other polypeptides include the transcriptional regulators Pol II, RNA helicase A, p53, STAT1, myc, and CtIP and repair mediators including Rad50 and BACH1 (11-20).

Several BRCA1-containing complexes have been purified using different methods. The functions of these complexes are likely to be

different. BRCA1 involvement in transcription is indicated by its associaton with the RNA polymerase II holoenzyme (holo-pol) and by activation of transcription by BRCA1 in cell free reactions (11,21-23). BRCA1 is associated with the chromatin-remodeling SWI/SNF complex, either in association with the holo-pol (22) or independent of Pol II (6). BRCA1 association with Rad50-Mre11-Nbs1 may contribute to repair of DNA damage. The BASC (BRCA1-associated genome surveillance complex) contains various proteins for DNA repair, including the Rad50 complex, cell cycle check point, and DNA replication factors (24). Since BASC is derived from a single-step immunoprecipitaion (IP) from unpurified nuclear extracts, it is unclear whether it represents multiple complexes or a single complex.

BRCA1 protein dynamically changes its subcellular localization dependent on the cell cycle or whether the genome has been damaged. In S-phase, BRCA1 localizes to discrete nuclear foci (25) but treatment with hydroxyurea (HU), ultraviolet (UV) irradiation, or gamma irradiation lead to dispersal of these BRCA1 foci (26). After HU and UV treatment, BRCA1

colocalizes with BARD1 and RAD51 in PCNA-containing, replication structures (27). After HU treatment or irradiation, BRCA1 forms a complex with Rad50, Mre11, and Nbs1 in discrete nuclear foci (irradiation-induced foci) (19,24). It is unknown whether these changes in subcellular position reflect changes in BRCA1 protein complexes.

In this study we identify that BRCA1 associated with three protein complexes in asynchronously cycling cells and that BRCA1 shifts to a fourth protein complex after treating cells with HU. We further characterize BRCA1 interaction with holo-pol. These data support a concept that the multiple processes with which BRCA1 is involved reflect multiple protein complexes with which it associates.

MATERIALS AND METHODS

Cell culture and Biochemical purification –HeLa-S3 cells and 293S cells were passaged in suspension culture using standard procedures. About 5 X 10^9 Cells were infected with recombinant adenovirus at an MOI of about 1-2

and cells were harvested 44 hours post infection. The purification from whole-cell-extracts by chromatography on a Biorex70 ion exchange matrix and sucrose gradient sedimentation have been described previously (21,22).

Adenovirus construction- HA-epitope tagged full-length and deleted mutants BRCA1 were inserted into AdEasy (Quantum Biotechnology, Inc.) shuttle vectors such that the BRCA1gene would be under the control of the CMV promoter. Full-length HA-epitope tagged BRCA1, to the amino terminus (HA-BRCA1) or to the carboxy terminus (BRCA1-HA), were subcloned from constructs in the pcDNA3 vector (25). The 1-302 deletion was constructed by digestion of Hind III and EcoR I from BRCA1-HA and then ligated insertion of the following forward (F) and reverse (R) linkers: F, 5'-AGCTTATAATGACCGGTG-3', and R, 5'-AATTCACCGGTCATTATA-3'. The 305-770 deletion was constructed by digestion of EcoR I and Kpn I from HA-BRCA1 and then ligated insertion of the following forward (F) and reverse (R) linkers: F, 5'-AATTCGGACCAAAGAAGAAGCGTAAGACCGGTCTGGTAC-3', and R, 5'-CAGACCGGTCTTACGCTTCTTCTTTGGTCCACCG-3'. The 775-

1292 deletion was constructed by digestion of Kpn I and Nhe I from HA-BRCA1 and then ligated insertion of the following forward (F) and reverse (R) linkers: F, 5'-CTGGTGGACCAAAGAAGAAGCGTAAGACCGGTG-3', and R, 5'-CTAGCACCGGTCTTACGCTTCTTCTTTGGTCCACCAGGTAC-3'. The 1527-1863 deletion mutant was generated by digestion of full-length HA-BRCA1 of pcDNA3-5' HA BRCA1 with Hind III and Sac I, and then this fragment was inserted into the AdEasy shuttle vector. With each shuttle vector construct, recombination occurred in bacteria to recover adenoviral genomic DNA with the BRCA1gene, and virus was recovered following transfection into 293A cells.

Immunoprecipitation- 400-500 µl of protein from sucrose gradient fraction or 150-180 µl of eluted protein from the Biorex70 column was immunoprecipitated with the specific antibody 12CA5 for HA-epitope, hSRB7, or Mre11. 450-750 µl of binding reactions were incubated with rotation for 4h-overnight at 4 °C in buffer H (20 mM Tris-OAc, pH 7.9/ 1

mM EDTA/ 5% glycerol)/ 0.12 M KOAc/ 0.1% Nonidet P-40/ 0.1 mM dithiothreitol/BSA (0.2 mg/ml)/0.5 mM phenylmethlsulfonyl fluoride in the presence of protein extract, 3-5 µl of antibody, and 20 µl of protein A beads. When affinity purified anti-SRB7 antibody was used, these steps are performed with or without antigenic peptide (0.1mg/ml). With all IPs, supernatant was removed, and protein beads were then washed three times using 450-800 ml of wash buffer (120 or 300 mM KOAc/ 20mM Tris-OAc, pH 7.9/ 0.1% Nonidet P-40/ 0.1mM dithiothreitol/ 0.5mM phenylmethlysulfonyl fluoride). For Western blot analysis, samples were subjected to electrophoresis in 5% or 6% SDS-polyacrylamide gels and immunoblotted using the indicated antibodies.

RESULTS

Identification of the hydroxyurea induced complex (HUIC).

We have previously identified two separate protein complexes containing BRCA1 in HeLa cell extracts (21,22). One complex was identified as the holo-pol and the second complex was a more massive

complex, which may have contained a nucleic acid component (22). A third BRCA1-containing complex has been described which contains Rad50-Mre11-Nbs1 along with BRCA1 (19) or containing other repair factors (24). Data have suggested that BRCA1 shifts its subnuclear localization and perhaps associates with new protein complexes in the S-phase in the cell cycle (25,28). In order to block cells in large-scale culture in S-phase, we treated HeLa-S3 cells with 10 mM hydroxyurea (HU) for 16 hours. We reasoned that, regardless of the position of a given cell in the cell cycle, this incubation time was sufficient to drive all cells into S-phase whereupon DNA replication is blocked due to depletion of deoxynucleotide triphosphates. Extracts were then prepared by standard procedures (22). When comparing asynchronous cell extracts with HU-treated extracts, we observed an increased abundance of cyclin A relative to cyclin E, suggesting that the HU-treated extract was indeed enriched for S phase proteins (Figure 1A). Extracts were subjected to ion exchange chromatography, using Biorex70 matrix, and comparison to our published observations from asynchronous extracts (22) revealed that BRCA1 and Pol II fractionation on

this matrix were unchanged by the HU treatment. Most of the BRCA1 eluted in the 0.6 M KOAc step elution, and a lesser amount of BRCA1 was observed in the 0.3 M KOAc step elution. Most of Pol II was observed in the 0.6 M KOAc elution (Figure 1B).

We next fractionated BRCA1 using sucrose gradient sedimentation. Whereas Pol II was observed to peak in the fractions consistent with the holo-pol in both extracts (+ and – HU), BRCA1 was shifted in its sedimentation pattern. In asynchronous cell extracts, BRCA1 is observed in two pools of protein, one in fractions 1-3 at the bottom of the gradient, and one pool in fractions 11-17 consistent with the holo-pol (Figure 1C, *top*). The HU-treated extract resulted in an observed shift in BRCA1 from these two complexes to a third complex with lower mass and which we call the HU induced complex (HUIC; Figure 1C). The BRCA1 content in fractions 1-3 was markedly decreased, and the BRCA1 content in fractions 11-15 was also diminished, but a new peak of BRCA1 protein was observed in fractions 17-21. The holoenzyme specific marker, cycC (29), eluted

primarily in fractions 9-15, consistent with those fractions containing the holo-pol (Figure 1C). It is thus inferred that this third peak seen only after adding HU is not associated with the holo-pol. While the observations with the asynchronous extracts are consistent with published observations (21,22), the HUIC has not yet been described.

In order to characterize further the HUIC, as well as other BRCA1-containing protein complexes, we established a system for overexpressing BRCA1 in preparative extracts.

Overexpressed BRCA1 copurifies with holo-pol.

Recombinant adenoviruses, which express full-length BRCA1 with an amino-terminal HA tag, were used to infect approximately 5X 10^9 293S cells in suspension culture. Whole-cell-extracts were prepared by standard procedures (22) and chromatographed on a Biorex70 ion exchange matrix and protein fractions were eluted in washes of increasing concentrations of potassium acetate (Figure 2A). Immunoblotting of eluted proteins for HA-

BRCA1 and for Pol II revealed that overexpressed BRCA1 co-fractionated in the 0.6 M KOAc elution similarly as observed with the holo-pol from HeLa cells (Figure 2B and ref. 21). This 0.6 M KOAc peak fraction was then subjected to sucrose gradient sedimentation (Figure 2C). Immunoblotting for HA reveals that HA-BRCA1 has two peaks of protein concentration, in fraction 5 and fractions 13-17. By contrast, Pol II has a broad peak with highest concentrations that correspond to the peak of HA-BRCA1 in fractions 13-17 (Figure 2C), suggesting that the peak of BRCA1 in Fraction 5 is a distinct pool of the protein and is not the holo-pol. Overexpression of BRCA1 did not result in significant aberrant pools of BRCA1, as this pattern of fractionation of BRCA1 is almost identical to the endogenous BRCA1 in HeLa cells (Figure 1 and ref. 22). The pattern of the overexpressed BRCA1 does result in sharp peaks on the sucrose gradient, and this will be shown to be due in part to the absence of the HUIC when the full-length protein is overexpressed (see below). In addition, overexpression of a similar BRCA1 protein, but HA-tagged on the carboxy-terminus, resulted in an identical purification over these two steps (data not shown).

To confirm that overexpressed HA-BRCA1 interacts with Pol II, we immunoprecipitated from sucrose gradient sample 14 using the monoclonal HA-specific antibody and compared with the equivalent fraction from uninfected 293S cells (Figure 2D). Pol II was purified in the sample with the overexpressed HA-BRCA1, but only a background level of Pol II was detected in the IP from the uninfected cells. These results showed that overexpressed epitope-tagged BRCA1 is associated with the holo-pol similarly as is the endogenous BRCA1.

The amino-terminal and carboxy-terminal regions of BRCA1 are important for association with the holo-pol.

Deletion mutants of BRCA1 (Figure 3A) were inserted into recombinant adenovirus and infected into 293S cells. Infected whole-cell-extracts were chromatographed on Biorex70 matrix and analyzed by immunoblotting using antibody specific for the HA-tag and Pol II. Unlike the tagged full-length BRCA1, the BRCA1 deletion mutants fractionated in

multiple fractions from the Biorex70 matrix. HA-BRCA1 (Δ305-770) and HA-BRCA1 (Δ775-1292) eluted primarily in the 0.6 M KOAc peak consistent with the wild type BRCA1. The carboxyl-terminal deletion mutant (Δ1527-1863) eluted equally in the flow through, 0.3 M KOAc and 0.6 M KOAc elutions. By contrast, the amino-terminal deletion mutant, BRCA1 (Δ1-302), was fractionated primarily in the 0.15 M KOAc flow through. In this sample, endogenous BRCA1 was eluted in the 0.6 M KOAc fraction, as is normally observed (data not shown). Pol II had unchanged fractionation in these samples with the exception that some Pol II eluted in the flow through fraction. Interestingly, western blots for Pol II in samples containing the carboxy-terminal deletion mutant (Δ1527-1863) required prolonged exposure to visualize Pol II. Thus we analyzed the unfractionated extract for total Pol II content and found that the amount of Pol II in the sample expressing the carboxy-terminal deleted BRCA1 was significantly decreased, about three-fold, relative to the other samples (Figure 3C). This result suggests that BRCA1 function is required for stability of Pol II.

Next, we tested whether these BRCA1 deletion mutants were associated with the holo-pol by immunoprecipitation (IP) using the holoenzyme-specific antibody directed against SRB7 (30). The antibody was affinity-purified, and the input protein was the Biorex70 0.6 M KOAc eluate (Figure 4). All samples are evaluated in threes: 10% input protein was in the first lane of each trio (lanes 1, 4, 7, 11, 14), and IP with SRB7 in the presence of its antigenic peptide was in the second lane of each set (lanes 2, 5, 8, 12, 15) and SRB7 IP in the absence of blocking peptide in the third lane of each set (lanes 3, 6, 9, 13, 16). The use of the antigenic peptide in the second lane of each was to control for the specificity of the immunoprecipitation. Immunoblots were stained using antibodies specific for the HA epitope to detect overexpressed BRCA1 (*top*), endogenous BRCA1 (*second from top)*, Pol II (*third panel*), SWI/SNF subunit BRG1 (*fourth panel*), and the BRCA1-interactor BARD1 (*bottom panel*). With the full-length HA-BRCA1 (lanes 1-3), we observed HA-BRCA1, Pol II, BRG1, and BARD1 all associated with the SRB7. The first three of these confirmed published results (22), but the identification of BARD1 associated

with the holo-pol has never been demonstrated. Rad50 was negative for immunopurification by SRB7-antibody (data not shown). In these samples (lanes 1-3), the endogenous BRCA1 and full-length HA-BRCA1 comigrated.

The deletion mutants HA-BRCA1(Δ305-770) and HA-BRCA1(Δ775-1292) were not different from the full length BRCA1 with regard to association with SRB7. Of interest in this analysis, since these mutants migrate faster than the endogenous BRCA1, we observed that these mutants competed with the endogenous BRCA1 for binding to the holo-pol. (Figure 4, lanes 4-9).

The amino-terminal deleted HA-BRCA1(Δ1-302) was not enriched in the 0.6 M KOAc fraction (Figure 4), thus this mutant was not significantly present in the input. Instead, endogenous BRCA1 was fractionated in the 0.6 M KOAc fraction and associated with the holo-pol (Figure 4, lanes 11-13). The carboxy-terminal deleted HA-BRCA1 was associated with the

SRB7 complex, but, surprisingly, the amount of Pol II in this complex was at background levels (Figure 4, lanes 14-16).

These results are summarized in Figure 5. BARD1 is an integral holo-pol component. Thus, the amino-terminal deletion mutant did not interact with SRB7 complex, because it lacks the domain that interacts with BARD1. It was surprising that the amino terminus of BRCA1 is more critical than the BRCA1 carboxy terminus for its association with the holo-pol since our experiments have demonstrated that the carboxy terminus of BRCA1 can bind to the holoenzyme complex (21,22). The carboxy-terminal deletion mutant can interact with SRB7 complex, because it contains the BARD1 interacting domain. Previous results in which deletion of the carboxy terminal 11 amino acids of BRCA1 resulted in diminished binding to the holo-pol (21) probably reflect a smaller fraction of the BRCA1 association with the holo-pol, since we observed relatively high levels of this mutant in the Biorex70 flow through fraction and in the 0.3 M KOAc protein peak (Figure 3B). It was surprising that the Pol II did not associate with the SRB7

complex containing BRCA1 (Δ1527-1863) (Figure 4), suggesting that the BRCA1 carboxy-terminus is involved in stabilizing the association of Pol II with the SRB factors or the stability of Pol II itself.

Characterization of the HUIC and association with BARD1

We next applied the overexpression of tagged BRCA1 to the analysis of the HUIC. After infecting 293S cells with full-length BRCA1 we treated the cells with 10 mM HU for 16 hours prior to extraction. Chromatography on Biorex70 was unchanged (data not shown), and analysis of the 0.6 M KOAc peak on sucrose gradient revealed that there was no shift of the HA-BRCA1 into the HUIC as was observed with the endogenous BRCA1 (Figure 6A, compared with Figure 2C). Overexpressing BRCA1 causes a G1 arrest and growth suppression (data not shown and ref. 31-33). Since the HUIC was observed in extracts enriched in S-phase of the cell cycle, we infer that overexpressing full-length BRCA1 might be incompatible with BRCA1 association with the HUIC. In contrast, HA-BRCA1(Δ775-1292), which did not suppress growth of cells (data not shown), did reveal three

peaks on sucrose gradient sedimentation analysis (Figure 6A), consistent with the presence of the HUIC in these samples. HU treatment decreased the BRCA1 content present in the Fraction 5 and holo-pol peaks and increased the BRCA1 content in fractions consistent with the HUIC (Figure 6A). This pattern is similar to that observed with endogenous BRCA1 in HeLa cells following HU treatment (Figure 1C).

Whether the HUIC was a derivative of the holo-pol was tested by immunoprecipitation with the affinity-purified SRB7-specific antibody (Figure 6B). Fractions 5, 17, and 25 containing HA-BRCA1(Δ775-1292) (from Figure 6A) were immunoprecipitated with the SRB7-specific antibody, and the blot was stained for the HA-epitope (Figure 6B). These fractions were chosen since they represent the peaks of the Fraction 5, holo-pol, and HUIC complexes. When analyzing fraction 17, which had sedimentation consistent with the holo-pol, significant purification of HA-BRCA1 was observed (lanes 4-6), indicating association of the HA-BRCA1(Δ775-1292) with the holo-pol as observed in Figure 4. By contrast, the HA-

BRCA1(Δ775-1292) in fraction 25, the HUIC containing fraction, was not at all associated with SRB7 (lanes 7-9). The very faint band in lane 9 probably resulted from contamination of the HUIC by the holo-pol, which sedimented in adjacent fractions. This result suggests that the HUIC is a distinct complex from the holo-pol. The results of the IP from fraction 5 were weakly positive (lanes 1-3), suggesting that the protein complex in this sample may be derived from the holo-pol.

BRCA1 has been reported to interact with the Rad50-Mre11-Nbs1 repair complex and also with BARD1 (7,19), and these proteins all copurify with the HUIC (Figure 6A). We tested whether the HUIC is the same as the BRCA1-Rad50-Mre11-Nbs1 complex by immunopurification using Mre11-specific antiserum. While the Mre11 antibody could purify Mre11 and Rad50, HA-BRCA1(Δ775-1292) or BARD1 were not detected (Figure 6C). Thus, the HUIC is not the same as the Rad50-containing complex. By contrast we have identified a BRCA1-associated protein in the HUIC. Anti-

HA epitope immunopurification from the HUIC containing samples revealed that BARD1 was present in this complex (Figure 6D).

BRCA1-Rad50-Mre11-Nbs1complex.

It has been reported that the Rad50-Mre11-Nbs1 complex associates with BRCA1 (19), but we did not observe this complex in HUIC fractions even though the proteins cosediment. A small pool of BRCA1 was observed in the 0.3 M KOAc elution of the Biorex70 column (Figure 2B), and most of the total Rad50 in the extract was in the 0.3 M KOAc elution (data not shown). The 0.3 M KOAc Biorex70 eluate was analyzed by sucrose gradient sedimentation (Figure 7A). Samples expressing full-length HA-BRCA1 were subjected to western blot analysis and stained for HA-epitope, Rad50, Mre11, and Nbs1. These patterns are similar with Rad50, Mre11, and Nbs1 sedimentation in fractions 15 to 23. HA-BRCA1 was present in these samples but also shifted to a higher molecular weight and fractionated in 13 to 23. This shift in BRCA1 content was likely due to contamination by holo-pol (data not shown). Next, we tested whether BRCA1 is associated with

Rad50 in this 0.3 M KOAc chromatographic fraction using anti-Mre11 antibody. In this fraction, full-length HA-BRCA1 was associated with Mre11 as was Rad50 (Figure 7B). Finally we tested whether HU, which causes damaged DNA, would stimulate association of endogenous BRCA1 with the Rad50-Mre11-Nbs1 complex. Although it has been reported that HU treatment causes a colocalization of BRCA1 and Rad50 complex (24), HU treatment did not stimulate BRCA1 association with the Rad50 complex (Figure 7C).

DISCUSSION

Here we report the separation of four BRCA1 containing complexes. Upon Biorex70 chromatography, the BRCA1-Rad50 complex elutes in the 0.3 M KOAc fraction and is thus readily resolved from the other three complexes. And by sucrose gradient sedimentation analysis of the Biorex70 0.6 M KOAc fraction, we purified the Fraction 5 complex, holo-pol complex, and the HUIC (summarized in Figure 8).

We identified the HUIC, and we demonstrated that it contains BARD1. The subnuclear localization of BRCA1 and BARD1 changes in S-phase following treatment with HU or UV to PCNA-containing replication structures (26,27). Kleiman and Manley found that BRCA1/BARD1/CstF represses the nuclear mRNA polyadenylation in vitro and that in cells treated with HU or UV mRNA 3' processing is transiently inhibited. CstF50 and PCNA interact strongly in a two hybrid screen (5). We suggest that the HUIC protein complex identified in this study likely functions as the protein complex responsible for the activity which represses of polyadenylation of mRNAs (4).

We observed an interaction between BRCA1 and BRG1 subunit of the chromatin remodeling SWI/SNF complex only in the context of the holo-pol. BRCA1 has been observed to associate with the SWI/SNF complex independent of Pol II and independent of BARD1 (6). We suggest that the multiple chromatography columns used in those experiments (6) fragmented the BRCA1-containing holo-pol leaving BRCA1 bound to SWI/SNF. The

strength of the purifications applied in this study is that interactions between BRCA1 and its key regulators, BARD1 and Pol II, remain intact.

The association of BRCA1 with the Rad50-Mre11-Nbs1 complex was not stimulated by HU. We anticipated that this association would be stimulated by HU since HU causes the DNA replication arrest with DNA gaps which would likely induce a damage response. Our result with no increase in the association between BRCA1 and the Rad50 repair complex following HU treatment is consistent with similar results from another laboratory after gamma-irradiation or methyl methanesulfonate treatment (19). Current data have not yet determined whether the BRCA1-Rad50-Mre11-Nbs1 complex we heve identified is the same as the BASC.

BRCA1-BARD1 interactions are demonstrated in the most abundunt BRCA1-containing complexes. As mentioned above, the redistribution of BRCA1 among protein complexes is consistent with changes noted in subnuclear localization (26,27). We observed clearly that BRCA1 content

shifted from holo-pol complex and Fraction 5 complex to the HUIC following HU treatment of cells. This shift was less apparent for the BRCA1-Rad50 complex. Is the HUIC a derived product from the holo-pol complex or from the Fraction 5 complex? We found that the HUIC does not contain SRB7, Pol II, and BRG1 (data not shown), and in sucrose gradient sedimentation of the 0.6 M KOAc elution BARD1 fractionated broadly throughout the gradient including two peaks consistent with the Fraction 5 complex and the HUIC. Moreover, we show that BARD1 is an integral component of the holo-pol for the BRCA1 association. It is possible that HU treatment leads to degradation of holo-pol and the Fraction 5 complexes leaving a residual BRCA1-BARD1-containing complex in the HUIC. This hypothesis is consistent with a model in which transcription functions in transcription coupled repair and recombination as surveillance for DNA damage (34). Encountering DNA damage, the BRCA1-BARD1 ubiquitin ligase activity destroys the holo-pol leaving the HUIC at the site of damage, and the HUIC would then recruit repair factors. Additionally, this hypothesis might support the decreased stability of Pol II after deletion of the carboxy

terminal domain of BRCA1, suggesting that this domain could possibly negatively regulate ubiquitin ligase activity.

BRCA1 is a dynamic protein since it is present in asynchronously cycling cells in three complexes, but following DNA damage and S-phase arrest the BRCA1 content shifts to a new protein complex, the HUIC. It is likely that BRCA1 protein participates in multiple cellular pathways by different functions in different complexes.

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Figure 1. **Identification of the hydroxyurea induced complex (HUIC). A,** Hydroxyurea blocks cell cycle in S-phase. Whole cell extracts from asynchronous HeLa cell culture (lane 1) or from HeLa cell culture blocked in S-phase by hydroxyurea (lane 2) were analyzed for the presence of the S-phase specific cyclin A (top) or the G1/S phase specific cyclin E (bottom). The migration of the 66kD molecular weight marker is indicated at the left. **B**, BRCA1 from hydroxyurea treated HeLa cells copurifies with Pol II on Biorex70 matrix. Whole-cell-extract from hydroxyurea treated cells was chromatographed on Biorex70 and immunoblotted for BRCA1 and Pol II. Samples represent load (L), flow through at 0.15 M KOAc (FT), protein peak from the 0.3 M KOAc wash (0.3), protein peak from the 0.6 M KOAc wash (0.6), and protein peak from the 1.5 M KOAc wash (1.5). **C**, Identification of hydroxyurea induced complex (HUIC) by sucrose gradient sedimentation. The protein peak eluted from Biorex70 at 0.6 M KOAc was subjected to sucrose gradient sedimentation. Immunoblots of these fractions were stained for BRCA1, Pol II large subunit, and cycC. In fraction 17-21 of HU treated sample, a new peak was observed. Fractions on the left (low

numbers) represent rapidly sedimenting complexes, and fraction on the right (high numbers) represent low molecular weight complexes.

Figure 2. **Overexpressed BRCA1 copurifies with the holo-pol. A**, Scheme for purification of adenovirus infected cell extracts. **B**, After infecting HA-tagged BRCA1 into 293S cells, whole-cell-extracts were chromatographed on Biorex70, and eluted proteins were analyzed by immunoblotting for the HA epitope and Pol II. **C**, The 0.6 M KOAc fractions were subjected to sucrose gradient sedimentation and fractions were immunoblotted for the HA epitope and the Pol II large subunit. **D**, HA-BRCA1 is associated with holo-pol. Sucrose gradient-purified holo-pol (fraction 14) was epitope-purified using the HA epitope-specific antibody (lane 4). The same fraction of an uninfected sample was used as control (lane3). In lanes 1 and 2, 10% of the respective input samples were analyzed.

Figure 3. **Fractionation of BRCA1 deletion mutants**. **A**, Design of BRCA1 deletion mutants with locations of HA-tag and locations of nuclear

localization signal (NLS). **B**, Immunoblots of eluted fractions from Biorex70 matrix. Blots were stained for the HA epitope (left panel) and the Pol II large subunit (right panel). **C**, Pol II decreased in cells infected with the carboxy-terminal deleted BRCA1. Immunoblot of whole-cell-extracts of parent 293S cells, full-length BRCA1 infected cells, and deletion mutants infected cells stained for Pol II large subunit.

Figure 4. **Association of BRCA1 deletion mutants with the Pol II holoenzyme complex.** Immunoprecipitaion by holoenzyme-specific antibody directed against SRB7, using 0.6 M KOAc Biorex70 eluate as inputs. All samples are evaluated in threes: 10% input protein is in the first lane of each trio (lanes 1, 4, 7, 11,14), IP with SRB7 in the presence of its antigenic peptide is in the second lane of each set (lanes 2, 5, 8, 12, 15), and SRB7 IP in the absence of blocking peptide in the third lane of each set (lanes 3, 6, 9, 13, 16). The top panel is stained using antibody specific for the HA epitope. The second panel is stained using antibody specific for the BRCA1. The third panel is stained using antibody specific for the Pol II

large subunit. The fourth panel is stained using antibody specific for the SWI/SNF subunit BRG1. The fifth panel is stained using antibody specific for the BRCA1-interactor, BARD1. All indicated bands migrated at positions consistent with their molecular weights.

Figure 5. **Model for interaction of BRCA1 deletion mutants with SRB7 complex.** The amino terminal deletion mutant lacks the domain that interacts with BARD1, and thus it can not interact with SRB7 complex. Instead, endogenous BRCA1 interacts with this SRB7 complex. The carboxy-terminal deletion mutant can interact with SRB7 complex, because it contains BARD1 interacting domain in the amino-terminus. In samples infected with the carboxy-terminal deletion mutant, Pol II did not associate with SRB7 complex and the amount of Pol II decreased. The carboxy terminus of BRCA1 is involved in stability of the association of Pol II with the SRB7 complex or the stability of Pol II itself.

Figure 6. **Characterization of the HUIC from HA-BRCA1(Δ775-1292) expressing cells. A**, Immunoblots of sucrose gradient of full-length HA-BRCA1 and HA-BRCA1(Δ775-1292) infected cells. Full-length HA-BRCA1 did not reveal the HUIC when treated with HU. HA-BRCA1 (Δ775-1292) infected cells showed three peaks, fraction 5, fraction 13-17 (holo-pol), and fraction 23-25, the HUIC. HUIC peak of cells infected with HA-BRCA1(Δ775-1292) became more abundant after HU treatment. In fractions that contain the HUIC, there were peaks of expression of BARD1 and Rad50. **B**, The HUIC is not associated with SRB7. Three sucrose gradient fractions, fraction 5, 17, and 25 of HA-BRCA1 (Δ775-1292) expressing cells were immunoprecipitated by pol-holo-specific antibody directed against hSRB7. Samples are arranged in threes with 10% input (In; lanes 1, 4, 7), SRB7 IP with peptide block (+; lanes 2, 5, 8) and SRB7 IP without peptide block (-; lanes 3, 6, 9). **C**, HUIC does not contain the Rad50 complex. Immunoprecipitation from the HUIC-containing samples in HU-treated cells infected with HA-BRCA1(Δ775-1292) using antibody specific for Mre11 reveal purification of Rad50 and Mre11, but not HA-

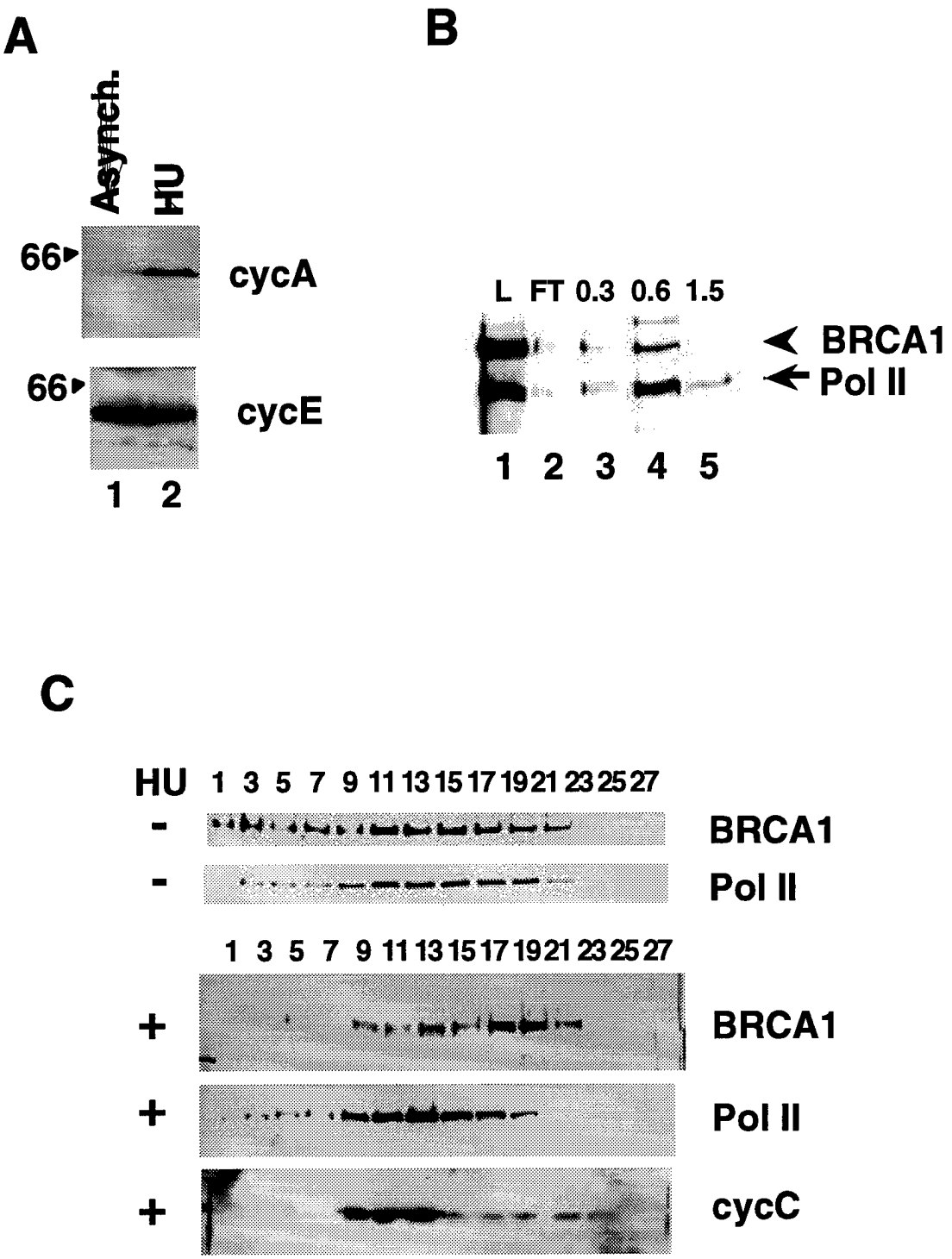
BRCA1(Δ775-1292) or BARD1. **D**, BARD1 is a component of the HUIC. The HUIC-containing samples in HU-treated cells infected with HA-BRCA1(Δ775-1292) (lanes 2, 4) or matched samples from uninfected HU-treated cells (lanes 1, 3) were immunoprecipitated with anti-HA antibody (lanes 3-4) and analyzed by western blot using antibody specific to BARD1 and HA epitope.

Figure 7. **Rad50 complex is associated with BRCA1 in the 0.3 M KOAc protein peak from the Biorex70 matrix. A**, BRCA1 in the 0.3 M KOAc Biorex70 protein peak copurifies with Rad50 complex on sucrose gradient sedimentation. The 0.3 M KOAc Biorex70 fraction from cells overexpressing HA-tagged full-length BRCA1 was subjected to sucrose gradient sedimentation, and fractions were immunoblotted for HA-epitope, Rad50, Mre11, and Nbs1. **B**, Immunoprecipitation, using anti-Mre11 antibody, purified from the Biorex70 0.3 M eluate containing full-length HA-BRCA1. Immunoblot was stained using antibody specific for the HA epitope and Rad50. **C**, Immunoprecipitation from the Biorex70 0.3 M eluate

chromatography of uninfected samples using antibody specific for Mre11. The samples were derived from cells not treated with HU (lane 1-3) or HU treated 293S cells (lane 4-6). Immunoblots were stained using antibody specific for BRCA1, Rad50, Mre11, and Nbs1.

Figure 8. **Four different BRCA1-containing complexes were resolved by the biochemical purification strategy.** Upon Biorex70 chromatography, the BRCA1-Rad50 complex elutes in the 0.3 M KOAc fraction. And by sucrose gradient sedimentation analysis of the Biorex70 0.6 M KOAc fraction, the Fraction 5 complex, holoenzyme complex, and the HUIC were resolved.

Chiba and Parvin, Figure 1



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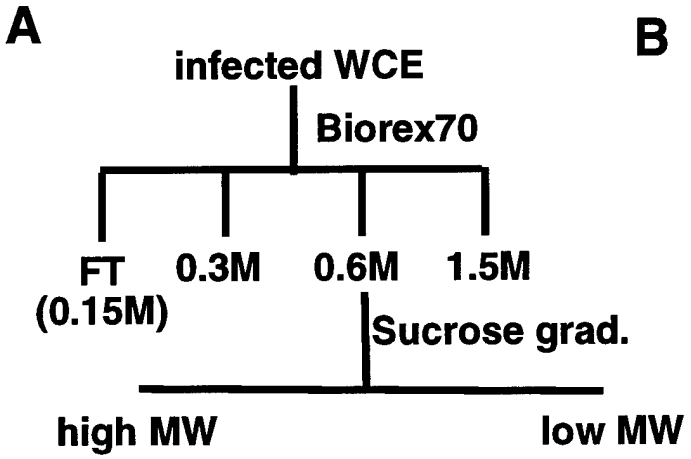

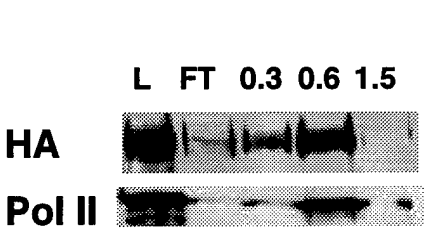

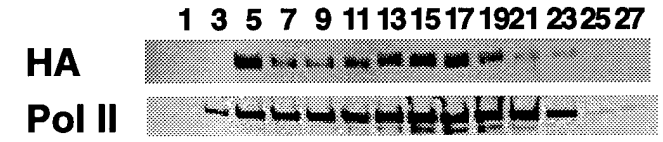

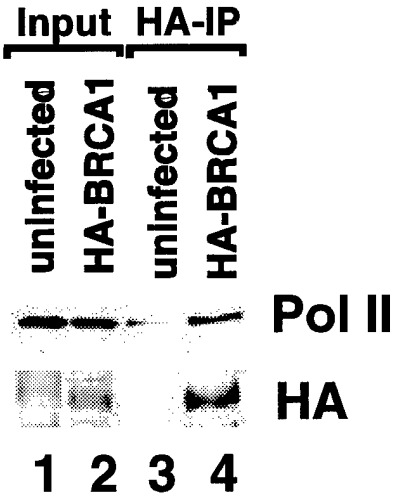

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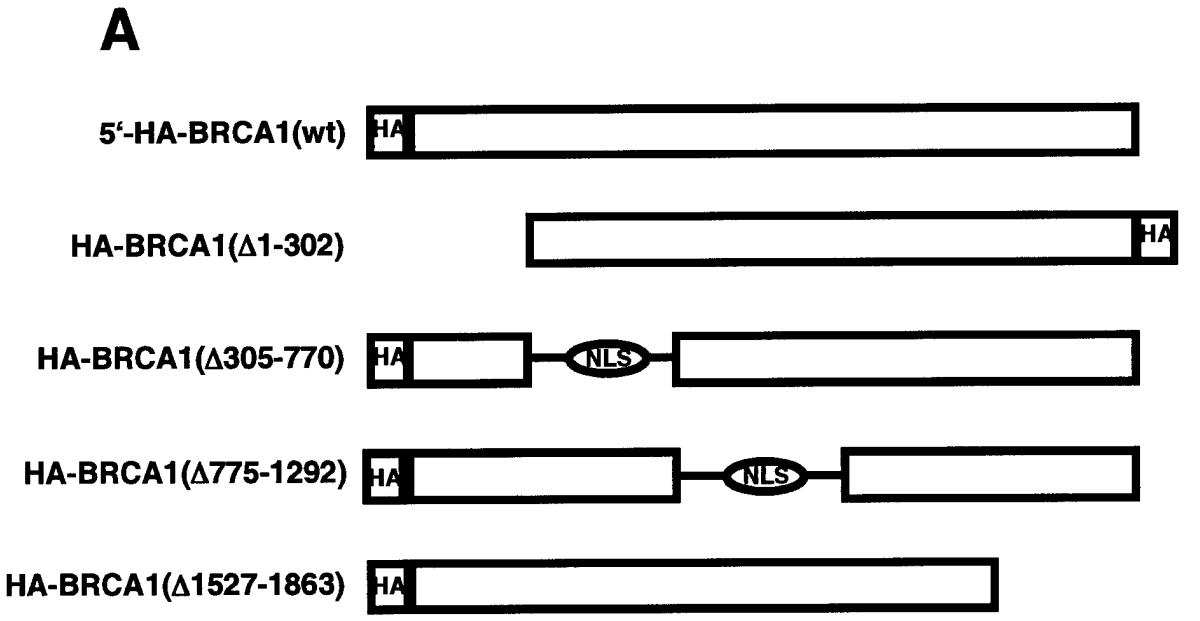

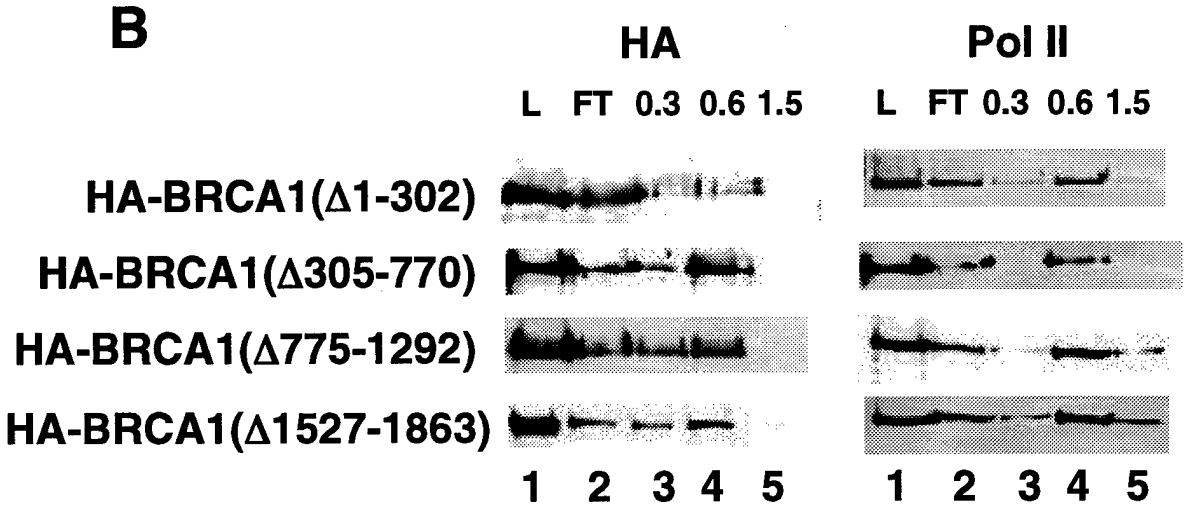

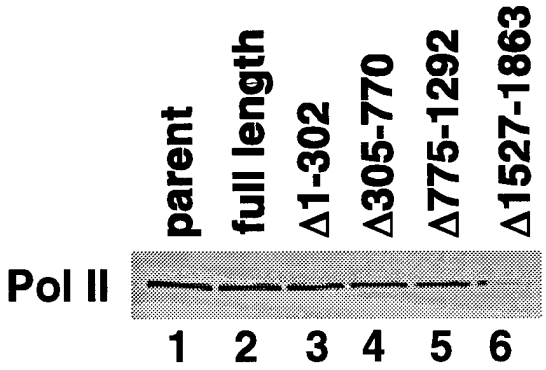

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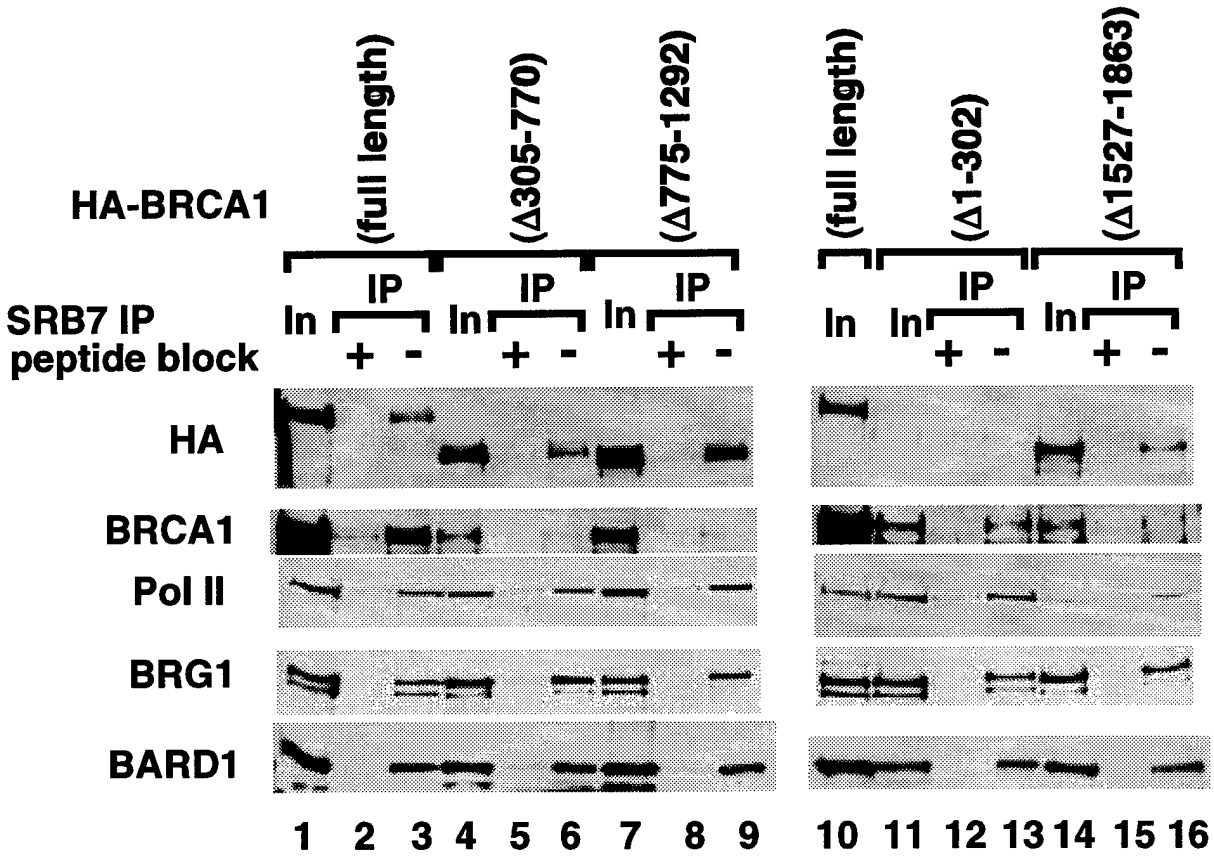
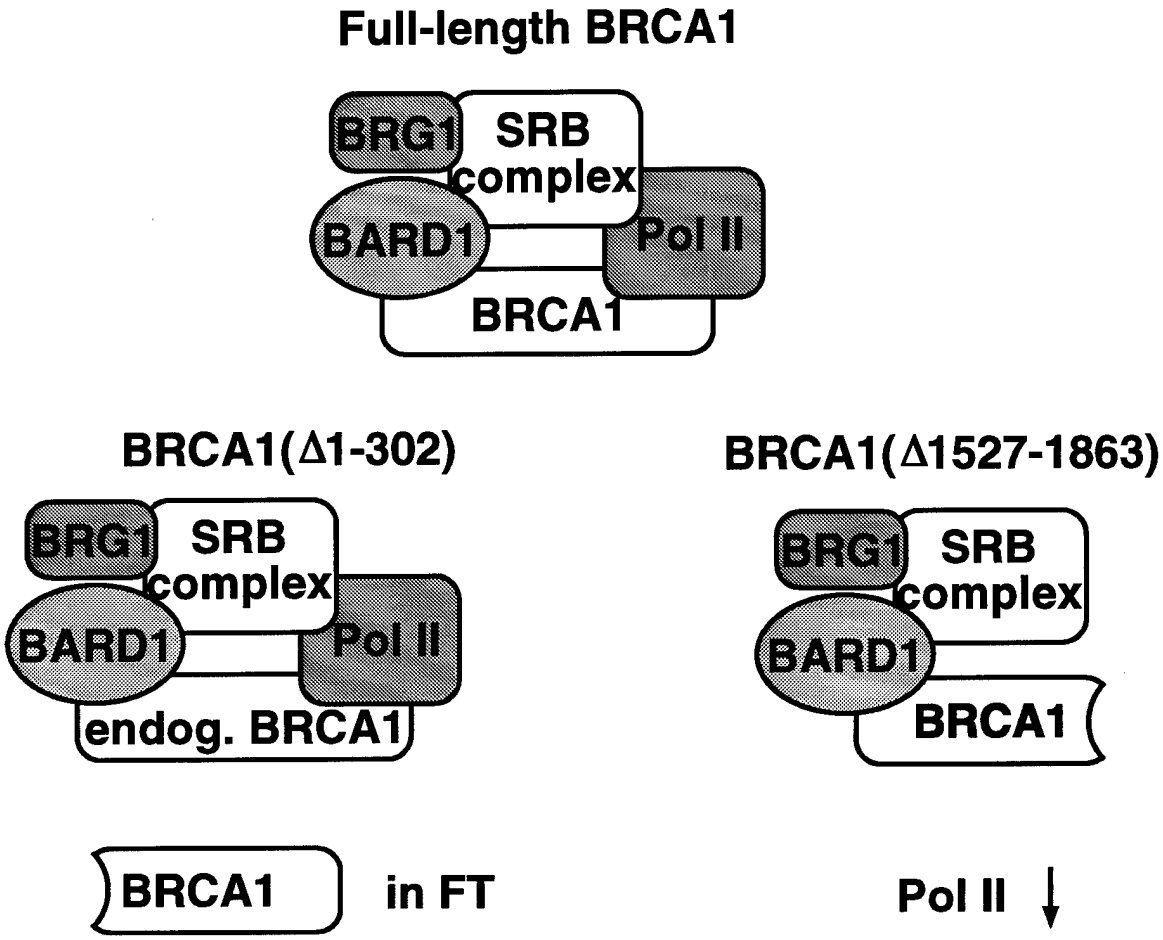
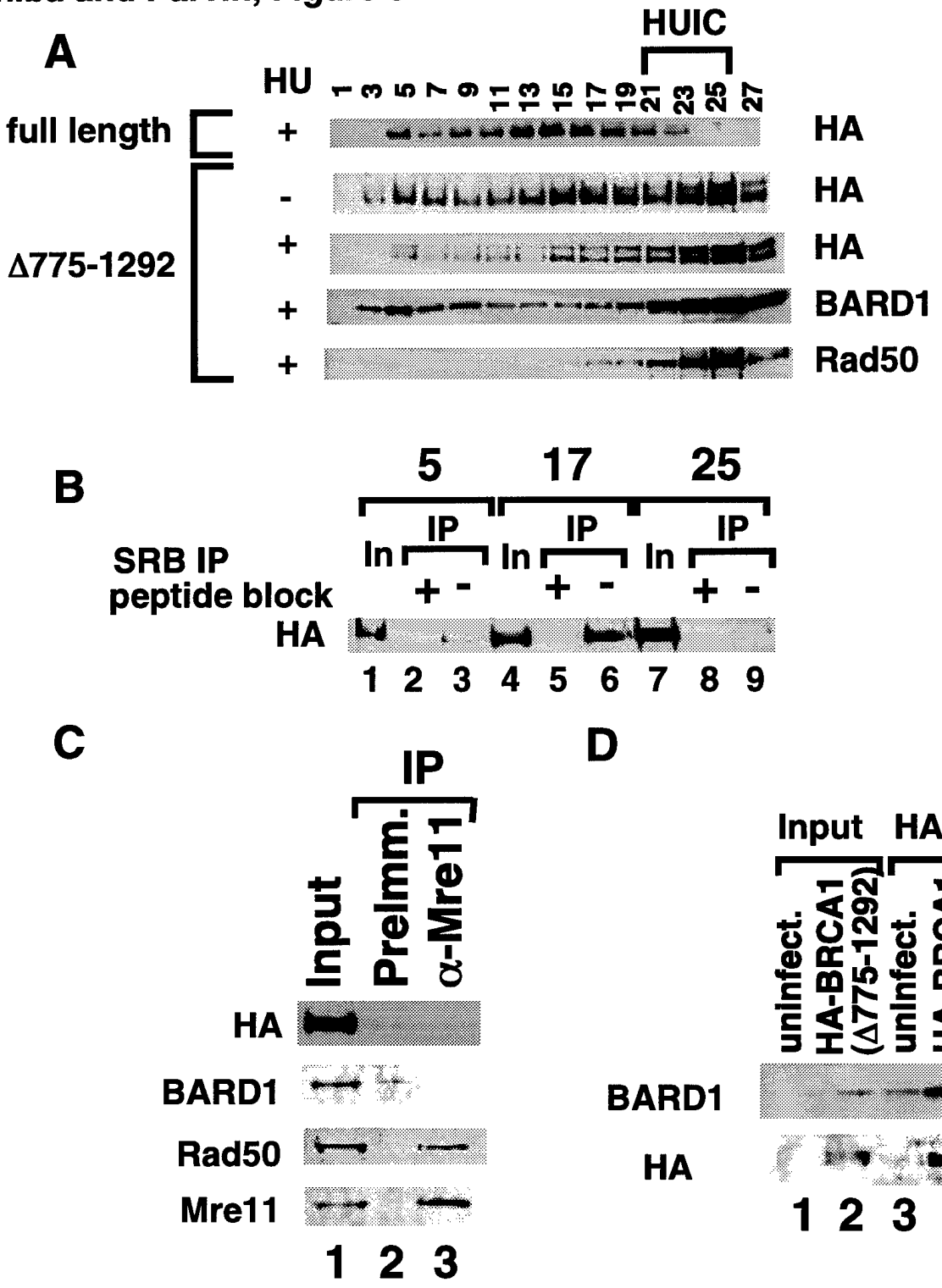


Chiba and Parvin, Figure 5



Chiba and Parvin, Figure 6



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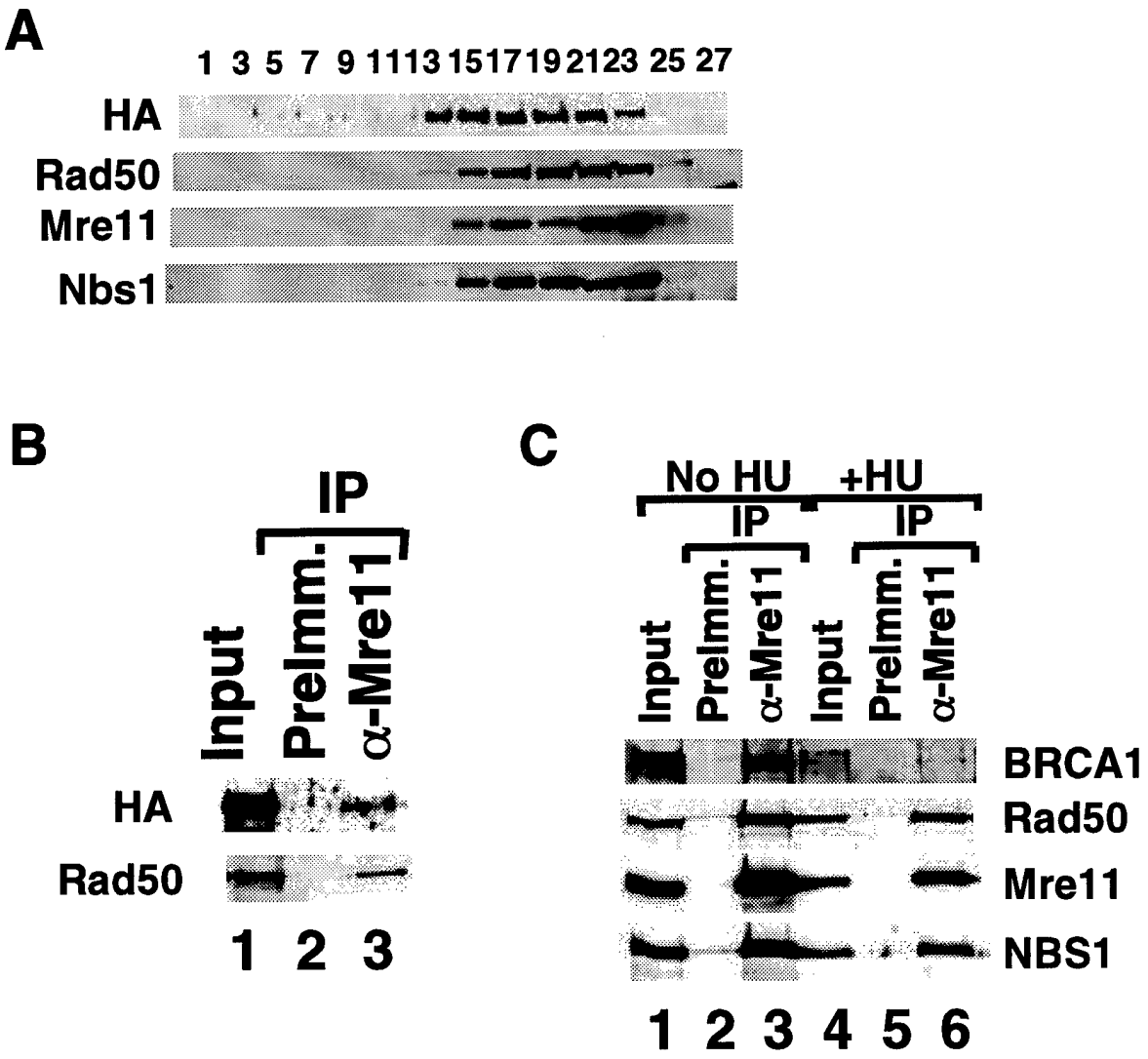

Chiba and Parvin, Figure 8